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Protein–alkaloid interaction in larval diet affects fitness in cactophilic *Drosophila* (Diptera: Drosophilidae)

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Drosophila koepferae and *Drosophila buzzatii* are closely related cactophilic species with overlapping distributions in Andean regions. Both species exploit necrotic tissues, and whereas the former breeds and feeds mostly in columnar cacti of *Trichocereus* and *Cereus* genera rich in secondary metabolites, the latter primarily exploits a less toxic host of the genus *Opuntia*. Although secondary metabolites have been related to the pattern of host exploitation, the microbial community associated with necrosis of cacti could also play a key role in the nutrition and/or alkaloid tolerance of the flies. We investigated the interaction between natural alkaloids and a yeast-protein supplement on both fly species raised in each type of cactus separately. We found that alkaloids reduced viability in both species, whereas a diet poor in protein reduced it only in *D. buzzatii*, especially when raised in *Trichocereus*. Concerning fitness traits that are related to adulthood, the addition of yeasts had positive effects, whereas the absence of yeasts resulted in strong detrimental effects. We present evidence of antagonistic effects on fitness and an interaction between alkaloids and proteins when these components are present in the diets of the flies.

ADDITIONAL KEYWORDS: alkaloids – cacti – *Drosophila* – genetic variation – nutrition – phenotypic plasticity – yeasts.

INTRODUCTION

The nutritional requirements of different insects are often fairly specific and include certain ratios between protein and carbohydrate for an optimal development (Behmer, 2009). Additionally, plants are often suboptimal food owing to the presence of allelochemicals and/or inadequate nutrient ratios (Schoonhoven *et al.*, 2005). Nevertheless, bacteria, yeasts and other microorganisms degrade plant tissue and could produce the nutritional requisites for insects that plants do not provide directly (Barbosa *et al.*, 1991; Schoonhoven *et al.*, 2005). Microorganisms (mainly yeasts) also contribute to the protein content of insect food intake, providing an adequate protein and carbohydrate ratio. However, yeasts contribute more than providing proteins; they also have important roles in detoxification of chemicals present in host plants and produce chemical signals used by insects to find food and oviposition sites (Barker & Starmer, 1999; Soto *et al.*, 2017).

Plants produce chemical defences, and herbivores and microorganisms develop countermeasures in response to these defences, in a dynamic of co-evolution often portrayed as an 'arms race' (Barbosa & Letourneau, 1988; Berenbaum, 1988; Mello & Silva-Filho, 2002). In order to understand how insect species with similar traits co-occur in such a complex environment, it is necessary to assess differential niche exploitation, because species that are ecologically very similar cannot coexist easily (Gause, 1934; MacArthur & Levins, 1967; Burns & Strauss, 2011). In this manner, phenotypic plasticity might be a cornerstone of adaptation, where genes can be viewed

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more accurately as 'potential resources' for alternative developmental pathways, allowing species coexistence and differentiation (Sultan, 2007, 2017).

The subgenus *Drosophila* offers a suitable framework for evolutionary ecology and genetic studies. There are several saprophytophagous species that breed on decaying plant material and feed upon components of the microbial community, such as bacteria, yeasts or moulds, with different degrees of specialization (Markow & O'Grady, 2008; Koch *et al.*, 2015). For instance, the *Drosophila repleta* cluster is one of the few cactophilic groups in the subgenus carrying out its life cycle on rotting cactus tissues (Ruiz & Heed, 1988; Oliveira *et al.*, 2012). Their ability to exploit cacti successfully has involved the acquisition of specialized detoxification mechanisms, allowing them to deal with complex chemicals, such as alkaloids and terpenoids, and with by-products of tissue decomposition, such as alcohols and esters (Fogleman & Danielson, 2001; Soto *et al.*, 2014; De Panis *et al.*, 2016). In this way, the cactus–yeasts–*Drosophila* system offers a complete picture of an integral trophic model in which to study trait adaptation, rapid diversification and specialization in a scenario of co-evolution (Fogleman & Danielson, 2001; Soto *et al.*, 2017).

Drosophila buzzatii (Patterson & Wheeler) and *Drosophila koepferae* Fontdevila & Wasserman are closely related cactophilic species of the *D. repleta* group with overlapping distributions in Southern South America, which breed and feed on the same hosts but with a marked differential use of the cactus species (Fontdevila *et al.*, 1988; Hasson *et al.*, 1992). *Drosophila buzzatii* mainly breeds on decaying *Opuntia* cacti, whereas *D. koepferae* exploits columnar cacti of the genera *Trichocereus* and *Cereus* as primary hosts. Both species display differentiated life-history, morphological and behavioural traits related to host specificity, such as oviposition preference, developmental time and body size, wing and genital morphology (Fanara *et al.*, 1999; Soto *et al.*, 2007, 2008, 2017). Recent studies that were focused on profiling the particular chemistry of host cacti [*Opuntia sulphurea* (G. Don in Loudon) and *Trichocereus terscheckii* (Britton & Rose)] revealed that the former species represents a less challenging environment than the columnar *T. terscheckii*, which produces highly toxic alkaloids, such as trichocereine and α-methylmescaline (de Panis *et al.*, 2016). Moreover, it has been postulated that these chemical differences exert divergent selection pressures, leading to different detoxification mechanisms between *D. buzzatii* and *D. koepferae* (Soto *et al.*, 2014; Padró *et al.*, 2018). However, differential toxicity among cactus hosts is only one possible factor affecting the patterns of host plant use.

Recent studies on the microorganismal community associated with cacti have identified four yeast species in rotting tissues of *T. terscheckii*, which correspond to a subsample of the eight species found in *O. sulphurea* (Koch *et al.*, 2015). Additionally, another recent study showed a strong preference of each fly species for its primary cactus host only when a particular cactophilic saprophytic biota was present (Soto *et al.*, 2017). These studies indicate the importance of incorporating the detritivore fauna in order to gain a better understanding of the complexity of the natural scenario.

Considering the yeasts only as a protein contribution, in *Drosophila melanogaster* it has been reported that protein rearing medium can modulate lifespan, egg-laying, reproduction rate and gene expression, among other traits (Min & Tatar, 2006; Gershman *et al.*, 2007; Lee *et al.*, 2008; Grandison *et al.*, 2009; Becher *et al.*, 2012). Additionally, Simpson & Raubenheimer (2001) found an interaction between nutrients and allelochemicals, where the detrimental effect of chemical components on locust performance disappeared when insects were provided with optimal levels of protein. The dependence on dietary protein for the capacity of an organism to mount an immune response has also been widely reported in various insects (Alaux *et al.*, 2010; González-Santoyo & Córdoba-Aguilar, 2012). However, little is known about the effect of nutritional factors on fitness traits in relationship to host plant use in cactophilic *Drosophila*.

The aim of this study was to evaluate the interaction between a protein supplement and the alkaloid fraction of *T. terscheckii* as modulators of the rearing conditions for larvae of *D. buzzatii* and *D. koepferae* by evaluating the response of different life-history and morphological traits. We focused on phenotypic plasticity between treatments but also on genetic variation and genotype–environment interaction (GEI) to assess the potential effect of a nutritious protein diet on strains of both flies. We hypothesize that in a nutritious medium, regardless of the rearing cactus, both flies will show higher performance. Accordingly, we predict that the positive effect of the nutritious protein medium will decrease or completely suppress the detrimental consequences of the toxic compounds, even when an extra dose of alkaloids is supplemented.

MATERIAL AND METHODS

DROSOPHILA STOCKS AND COLLECTION OF TISSUE FROM CACTI

We used three isofemale lines of both *D. buzzatii* and *D. koepferae* maintained in standard rearing medium (150 g dried potato rehydrated with a Nipagin solution 0.02 grams per liter of water and 20 g of commercial yeasts) and in controlled conditions (12 h–12 h light–dark photoperiod at 25 °C) for a year before the onset of the experiments. Each line was obtained from the

progeny of a wild-caught inseminated female collected in a natural population from northwestern Argentina (Valle Fértil, 30°38′4″S, 67°28′6″W; for further details, see Soto *et al.*, 2010).

At the same location, we also collected fresh pieces of the host cacti, *T. terscheckii* and *O. sulphurea*. We extracted the chlorenchyma of both cacti, homogenized it in a blender and stored it at −80 °C until the beginning of the experiments. In addition, we extracted the alkaloid fraction from *T. terscheckii* chlorenchyma, yielding a concentration of 0.9322 g/mL (for extraction details and alkaloid profiles, see de Panis *et al.*, 2016).

Experimental design

The design included two rearing conditions (Supporting Information, Table S1): the base rearing cacti and the additives. *Trichocereus terscheckii* (TR) and *O. sulphurea* (OP) were the two cacti used. 'Without any addition' (W), 'with commercial yeast protein added' (P) and 'with the addition of commercial yeast and the alkaloid fraction extracted from *T. terscheckii*' (A) were the three different diets used. We separated cactus tissue into three fractions to prepare each combination of both rearing conditions (treatments). The first fraction was mixed with 0.8 mL EtOH per 100 g of cactus (controlling for the solvent effects of the latter treatments mentioned below) and then reserved (obtaining treatments TR-W and OP-W). The same amount of EtOH as the first plus 3.45 g of killed yeast per 100 g of cactus was added to the second fraction (treatments TR-P and OP-P). To the last fraction was added with the same amount of killed yeast as for the second fraction, plus alkaloids (dissolved in EtOH) per 100 g of cactus: 0.4 mL of the alkaloid extract (0.9322 g/mL) plus 0.4 of EtOH was added to *T. terscheckii* rearing medium (which has a native concentration of highly toxic alkaloids of 0.4 mg/g), and 0.8 mL of the alkaloid extract was added to *O. sulphurea* rearing medium (which has a native concentration of slightly toxic alkaloids of 0.05 mg/g), resulting in treatments TR-A and OP-A respectively.

We placed adults of each isofemale line in rearing chambers with a Petri dish containing egg-laying medium (agar 2%). After 24 h, we removed the Petri dish from the chamber, checked for the presence of eggs and kept it for another 12 h until larval hatching. For each isofemale line, we transferred batches of 50 larvae from the respective Petri dish to vials containing 6 g of the corresponding treatment. Five replicates (vials) were set for each combination of line and treatment.

We collected the emerged adults every 24 h, counted them to evaluate larval viability (LV) in each vial, and estimated the developmental time (DT) as the time elapsed since the seeding of the larvae until adult emergence. To study the morphological response to alternative treatments, we removed the left wing of each fly (854 females and 868 males; for more details, see Supporting Information, Table S2), mounted them on slides with DPX and photographed them at ×40 magnification using a digital camera attached to a microscope (Nikon E200).

Wing morphology was analysed by separating size from shape variation. We located ten landmarks in the digitalized image of each wing using TpsDIG2 (Rohlf, 2015; for landmark configuration, see Carreira *et al.*, 2008). Wing size was estimated through the centroid size (Zelditch *et al.*, 2012). In contrast, variation in wing shape was estimated by generalized Procrustes analysis (Gower, 1975; Rohlf & Slice, 1990; Zelditch *et al.*, 2012), and then we performed principal components analysis to summarize the information on this trait.

Considering that wing size is a good proxy of body size (Hallas, 2002; Carreira *et al.*, 2006), we estimated the ratio between wing size and DT (Size/DT) per replicate and used it in all subsequent analyses as a performance index. Wing size is expected to be positively correlated with DT, and deviations above the average could be viewed as better performance (larger body size with rapid development) than deviations below the average (smaller body size with longer development).

Statistical analysis

To analyse the effect of the treatments, we applied a linear mixed model with a binomial distribution (GLMM) on viability and with normal distribution (LMM) on the Size/DT ratio for each species. One fixed environmental effect was tested: treatment, i.e. the combination of cacti and diets. The random effect of the line factor was also tested with a random interaction (line*treatment) to assess GEI. In cases of overdispersion, individual (i.e. observation level) was added as an extra explanatory random effect (Harrison, 2015). In the case of viability, to estimate significance of the fixed effect, we performed a parametric bootstrap test with 1000 iterations per parameter (Halekoh & Højsgaard, 2014), whereas we used the Satterthwaite approximation (which provides the best control of type 1 error for LMMs; Luke, 2017) with the Size/DT ratio.

We performed post hoc pairwise comparisons (Tukey's test) between levels for the treatment effect. For both GLMM and LMM, we tested the significance of the variance of random effects by a likelihood ratio test (Bolker *et al.*, 2009). Although there is no consensus on how to estimate the variance components for complex models (i.e. the contribution of each effect in models with crossing random effects; Nakagawa *et al.*, 2017), we extracted the explained variance of each random effect by comparing nested models with and without

the effect of interest to quantify the contribution of each random effect to total variation.

Lastly, we performed a *K*-means clustering (Hartigan & Wong, 1979) in order to search any possible conformational grouping and whether these are attributable to any of the sources of variation analysed. Additionally, we used two searching methods to determine the number of means for the clustering analysis: visual inspection of the first two principal components (non-scaled data) and gap statistics for scaled principal components (Tibshirani *et al.*, 2001). Moreover, we performed a distance (Euclidean) permutation test to assess the difference between the treatment levels (10 000 iterations per analysis), analysing each species and sex independently. The *P*-values were adjusted with the Holm method (Holm, 1979).

Analyses were performed in R statistical software (R Development Core Team, 2017). The packages *lme4* (Bates *et al.*, 2015) and *afex* (Singmann *et al.*, 2015) were used to perform GLMM, LMM and *P*-value estimations; *emmeans* (Lenth, 2018) and *multicomp* (Hothorn *et al.*, 2008) were used to perform post hoc analyses; and *geomorph* (Adams *et al.*, 2017) was used for morphometric analysis.

RESULTS

VIABILITY

The principal results of the viability analyses are reported in Table 1. Both *D. buzzatii* and *D. koepferae* showed significant differences among treatments. Post hoc analyses showed a higher viability of both species when raised in *Opuntia* than in *Trichocereus*. The addition of alkaloids resulted in lower viabilities in both species, mainly in *Trichocereus*. However, although there were no significant differences in viability between flies of both species reared in medium with protein and those grown in OP-W medium, the treatment TR-W resulted in the lowest viability for *D. buzzatii*, but also had a detrimental effect in *D. koepferae* (Fig. 1A, B).

The random effects line and GEI contributed 4.7 and 14.6% of variation, respectively, to the whole model in *D. buzzatii* (Table 1; Fig. 1C). In contrast, the significant effect of line and GEI explained 30.2 and 20.5% of variation, respectively, in *D. koepferae* (Fig. 1D). *Drosophila koepferae* lines showed 25.52% more phenotypic variation than *D. buzzatii* lines, although both species presented a similar contribution of the GEI to total variation.

RATIO OF WING SIZE TO DEVELOPMENTAL TIME

Principal results of the analyses of Size/DT ratio are shown in Table 1. Both species showed significant differences among treatments, where TR-W caused a significant reduction of Size/DT values in comparison to the rest of the treatments (Fig. 2A, B). Also, the addition of protein in both species resulted in similar Size/DT values between cacti for each diet (i.e. with and without alkaloids) and between diets in each cactus (except for the difference between TR-A and OP-A for *D. koepferae*).

More than 40% of the total variance was explained by the variation among genotypes within each species (Fig. 2C, D). Nevertheless, variation explained by GEI was more noticeable in *D. buzzatii* (39.46%) than in *D. koepferae* (23.38%; Table 1; Fig. 2C, D).

WING SHAPE

We selected three means for *K*-means clustering analysis after a visual inspection (obtaining ~97% of

Table 1. Principal results of the analyses of viability and Size/DT; estimator and degrees of freedom (d.f.) are shown for fixed and random effects

			Drosophila buzzatii		*Drosophila koepferae*	
Trait	Effect	Type	d.f.†	Estimator	d.f.†	Estimator
Viability	Treatment	Fixed	5	13.3*	1	13.78*
	Line	Random	2 (1)	8.64**	2 (1)	95.21***
	GEI	Random	5 (20)	27.71^{ns}	5 (20)	64.53***
Size/DT	Treatment	Fixed	5 [2]	161.62**	5 [2]	50.1*
	Line	Random	2 (1)	55.31***	2 (1)	95.65***
	GEI	Random	5 (20)	52.08***	5 (20)	50.87***

The significance of fixed effects was estimated by parametric bootstraps and the Satterthwaite approximation for viability and Size/DT, respectively, and the significance of all random effects was estimated by likelihood ratio tests.
Abbreviations: GEI, genotype–environment interaction; Size/DT, wing size/developmental time.
†In parentheses: difference of residual degrees of freedom between nested models. In square brackets: residual degrees of freedom obtained from the Satterthwaite approximation.
$^{ns}P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$. Values were corrected by the Holm method (Holm, 1979).

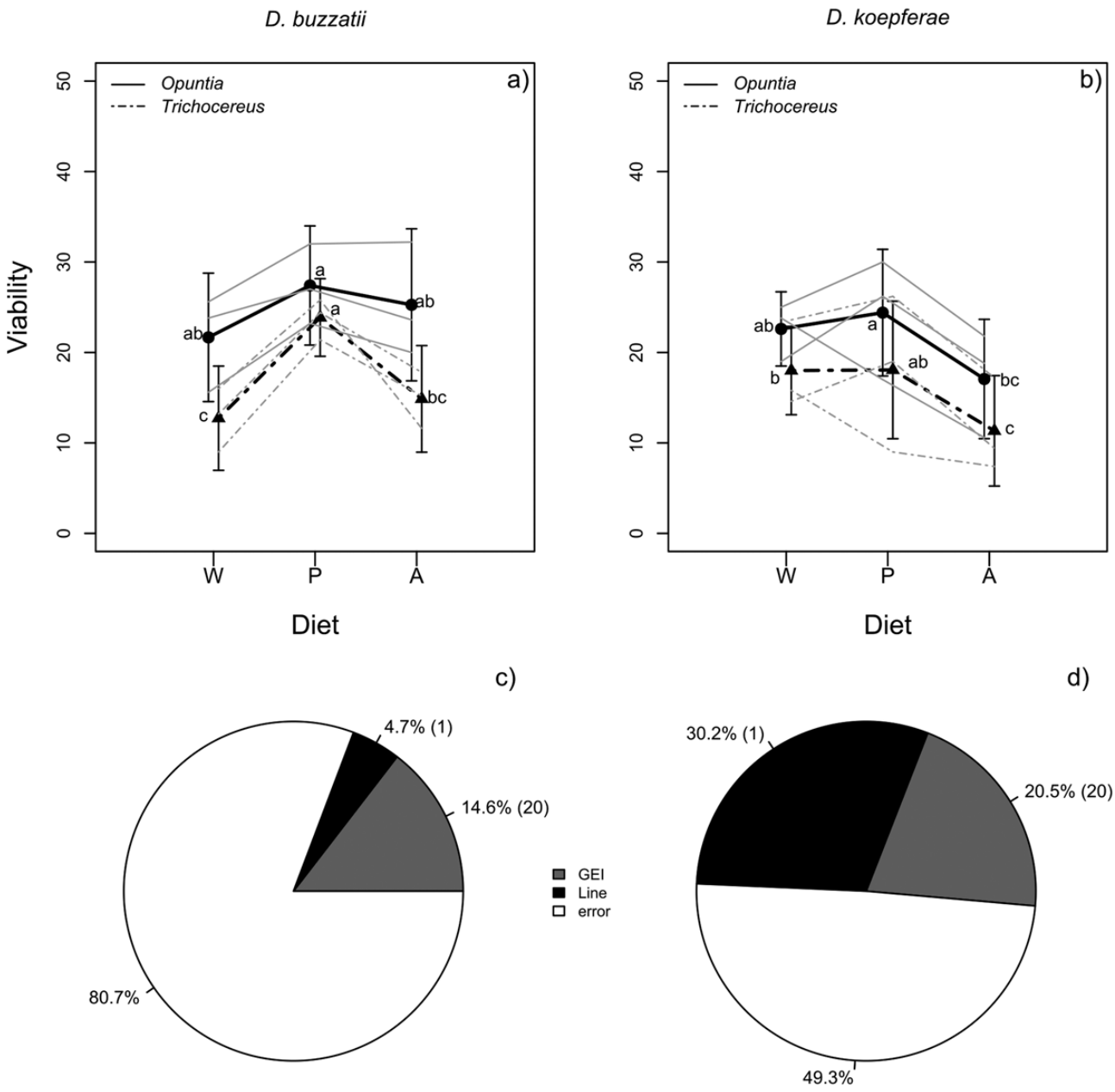

Figure 1. Viability data statistics of *Drosophila buzzatii* (left panels) and *Drosophila koepferae* (right panels) expressed as the number of emerged individuals. A, B, mean values and standard deviations (black lines) are shown in *Opuntia suphurea* (continuous lines) and *Trichocereus terscheckii* (dashed lines) for the following diet categories: without any addition (W), with addition of protein (P) and with addition of protein and alkaloids (A). Grey lines correspond to different isofemale lines. C, D, percentage of variance explained by each random effect and the degree of freedom of the residuals between nested models (in parentheses). Abbreviations: error, unexplained variance; GEI, genotype–environmental interaction (line*treatment); Line, isofemale lines. In A and B, different letters denote significant differences between treatments.

correct assignment; Supporting Information, Table S3) and six means through the gap statistic method (obtaining 96.6% of correct assignment; Supporting Information, Fig. S1; Table S4). Both groups selected showed a matching pattern: the main differences for wing shape were not among treatments but among isofemale lines. Indeed, with three means, the total of the individuals of a *D. koepferae* line and most individuals of one *D. buzzatii* line (99%) were included in the first and second cluster, respectively, capturing differentiable wing conformations, and the rest of the isofemale lines were in the third cluster (Fig. 3).

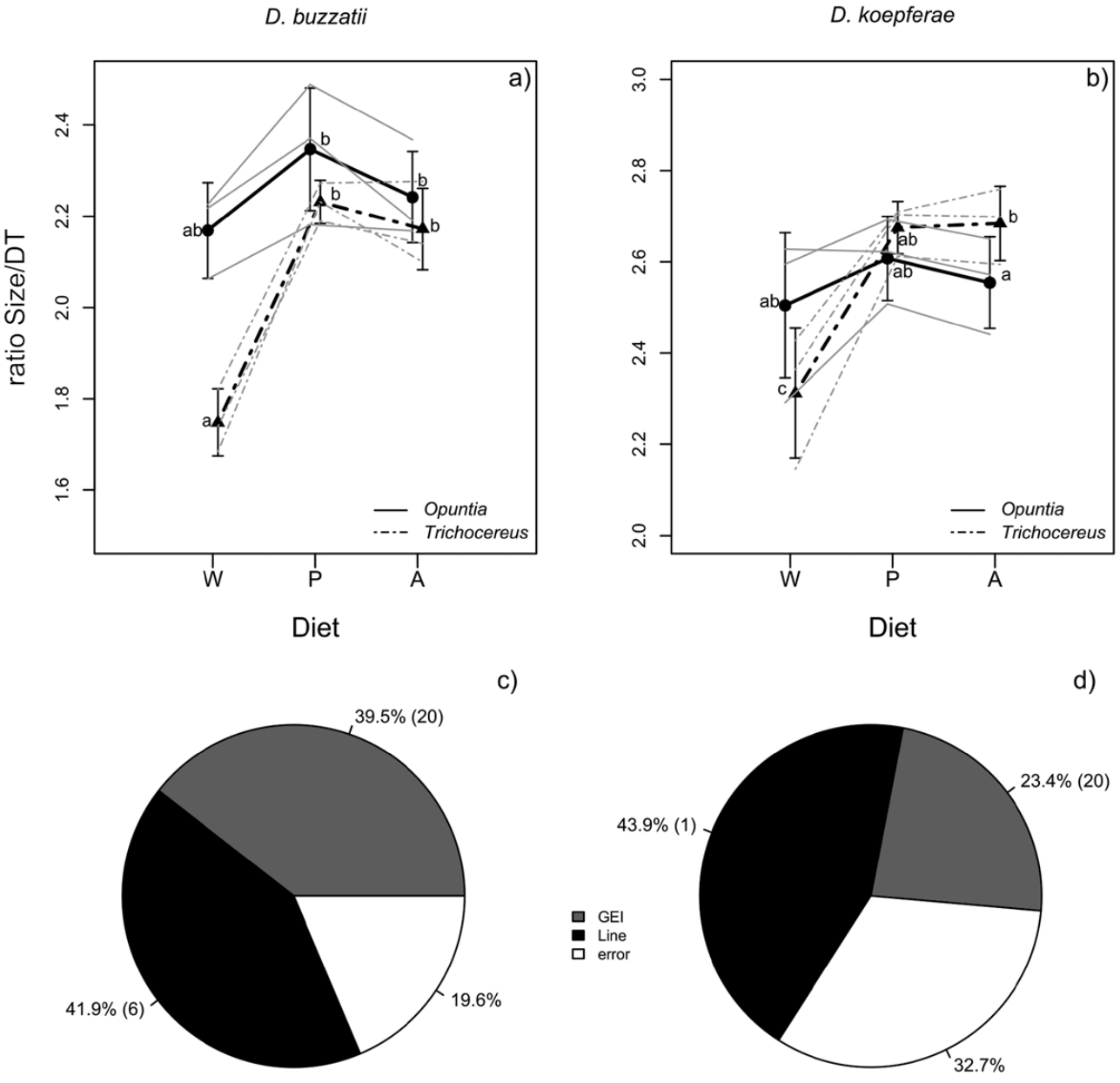

Figure 2. Wing size/developmental time (Size/DT) data statistics of *Drosophila buzzatii* (left panels) and *Drosophila koepferae* (right panels). A, B, mean values and standard deviations (black lines) in *Opuntia suphurea* (continuous lines) and *Trichocereus terscheckii* (dashed lines) are shown for the following diet categories: without any addition (W), with addition of protein (P) and with addition of protein and alkaloids (A). Grey lines correspond to different isofemales lines. C, D, percentage of variance explained by each random effect and the degree of freedom of the residuals between nested models (in parentheses). Abbreviations: error, unexplained variance; GEI, genotype–environmental interaction (line*treatment); Line, isofemale lines. In A and B, different letters denote significant differences between treatments.

Moreover, the wing conformation of each isofemale line was captured by each cluster when we selected six means for clustering analysis.

Even with the great variation among isofemale lines, we obtained significant differences among treatments when testing them through permutation tests. We observed a clear pattern of pairwise differences for both sexes in *D. buzzatii*: both treatments without protein (i.e. TR-W and OP-W) resulted in significantly different wing conformations with respect to the other treatments, but the protein treatments did not show any difference between them (Table 2). In contrast,

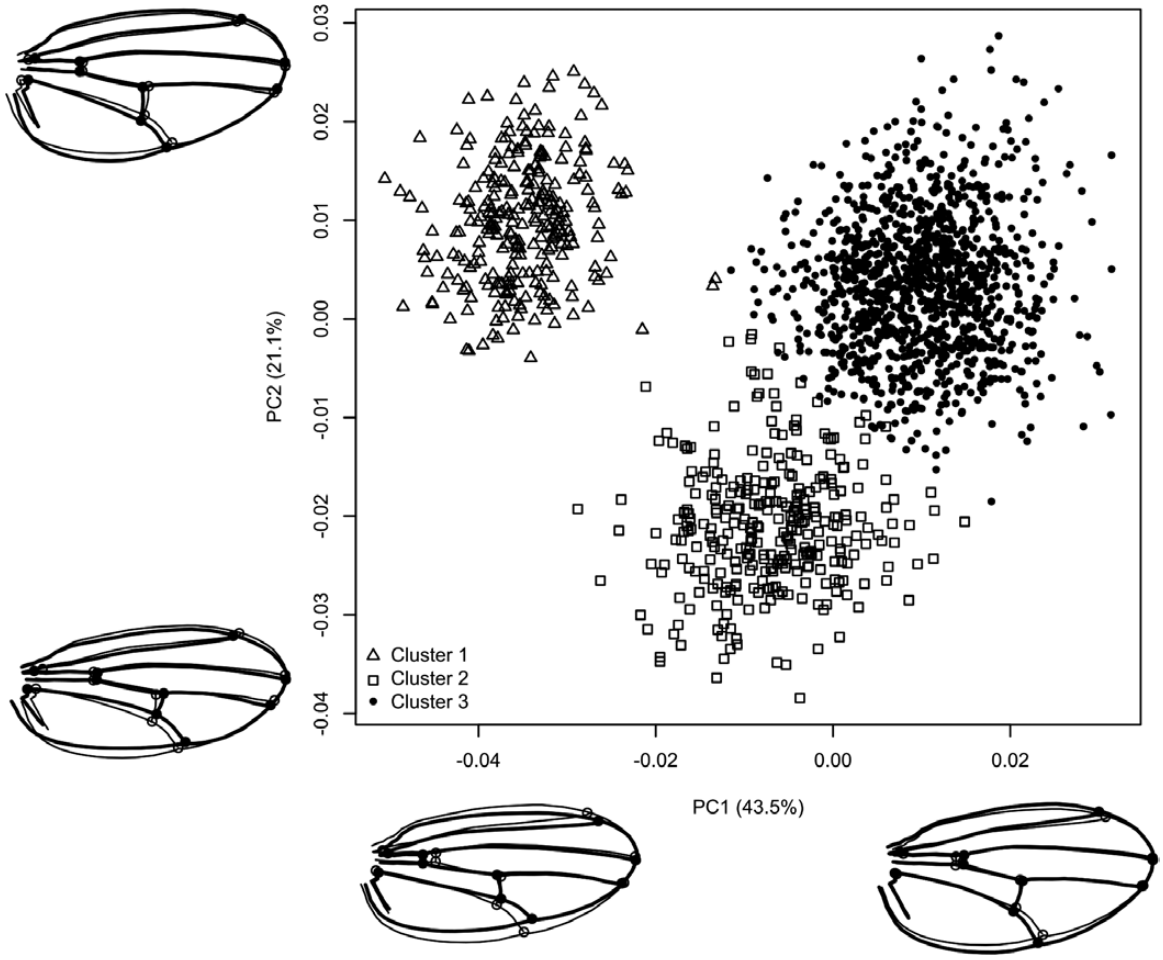

Figure 3. Wing shape represented by the first two principal components (PC1 and PC2), which together explain 64.6% of total phenotypic variation. Representation of different individuals (circles, squares and triangles) comes from *K*-means clustering analysis, where cluster 1 (triangles) corresponds to a whole line of *Drosophila koepferae* and one individual of another line of this species, cluster 2 (squares) corresponds to most individuals of a line of *Drosophila buzzatii* and some individuals of other lines of both species, and cluster 3 (circles) corresponds to the remainder of the individuals (most individuals of two lines of each species). Marginal plots represent changes in wing shape along each principal component with respect to the mean shape. Changes have been magnified two times for better appreciation.

D. koepferae showed significant differences for wing shape only for females, with the main differences being between cacti (although TR-W also showed significant differences from the rest of the treatments). Although we did not find any significant difference in *D. koepferae* males, differences were qualitatively the same as in females.

DISCUSSION

We found an interaction between the nutritional and toxic properties of the rearing medium that affected different phenotypic traits in *D. buzzatii* and *D. koepferae*. The response of both flies to the different breeding mediums was species-specific with few coincident patterns, highlighting the need to explore in depth on different traits the relationship between nutritional composition present in each cactus and the native toxicity of the medium.

Soto *et al.* (2014) have also shown that in a similar artificial breeding medium (i.e. *Opuntia* with alkaloids), *D. koepferae* attained low larval viability, postulating the possible importance of the nutritional components of its primary host. Based on the present study, it is possible to point out that the nutritional characteristics of *T. terscheckii* were not sufficient to compensate for the chemical stress caused by increased levels of alkaloids [twice as high as described by Soto *et al.* (2014) and twice the native concentration]. In disagreement with that previous work, we showed that the effect of alkaloid addition on viability was

Table 2. Euclidean distance matrix of wing shape between treatments: A, *Drosophila buzzatii*; and B, *Drosophila koepferae*

A	OP-A	TR-A	OP-P	TR-P	OP-W	TR-W	
OP-A		6.5	5.13	6.14	6.19	9.81**	M
TR-A	5.21		5.81	3.88	8.75*	9.38**	A
OP-P	5.23	6.24		5.29	7.98*	10.32**	L
TR-P	6.98	3.82	6.31		9.01**	9.15**	E
OP-W	8.75**	8.39*	7.56*	9.08**		10.08**	
TR-W	11.79**	10.48**	10.89**	9.47**	9.46**		
	F E M A L E						
B	OP-A	TR-A	OP-P	TR-P	OP-W	TR-W	
OP-A		8.33	3.86	7.76	4.92	10.14	M
TR-A	15.6**		8.17	3.74	7.29	8.27	A
OP-P	4.07	14.91**		7.13	3.79	9.38	L
TR-P	16.51**	2.5	15.83**		7.76	7.08	E
OP-W	6.21	12.71**	6.08	13.53**		9.92	
TR-W	13.97**	11.35*	12.88**	11.42*	10.17*		
	F E M A L E						

*$P < 0.05$, **$P < 0.01$ and *** $P < 0.001$. Values without asterisks indicate $P > 0.05$. Values were corrected by the Holm method (Holm, 1979).

mainly detrimental in *D. koepferae* regardless of the cactus. *Drosophila buzzatii* also showed lower viability when raised in *Trichocereus* with addition of alkaloids and in *Trichocereus* without any addition, showing a better performance in *Trichocereus* supplemented only with protein and in its primary host, *O. sulphurea*, regardless of the diet. However, our results also showed great genotypic variation, with a differential response in lines of *D. koepferae* raised in alternative treatments, suggesting a strong environmental dependence effect of the genotype.

Developmental time is a trait for which interpretation is not straightforward. In the same rearing conditions, it is expected that flies with a longer developmental time will be larger than flies with rapid development (Atkinson, 1994; Stearns, 2000; Cortese *et al.*, 2002). However, there is a trade-off between body size and developmental time, which are directly and inversely related to fitness, respectively. In this sense, large flies could have higher fecundity rates, increased lifespan and numerous ovarioles, among other traits (Santos *et al.*, 1992; Lefranc & Bundgaard, 2000; Reeve *et al.*, 2000; Speakman, 2005). In contrast, rapid development is related to higher population growth rate, predation avoidance and rapid escape from desiccation, among other things (Arendt, 1997; Gefen *et al.*, 2006; Krams *et al.*, 2016). Considering that larger flies with rapid development show higher fitness than smaller flies with longer development, the Size/DT ratio is expected to be correlated positively with fitness. Accordingly, our results suggest that protein buffers the detrimental effect of alkaloids; both species showed the lowest values of Size/DT in TR-W, but responded in a similar manner with the addition of protein (both treatments: protein alone and protein with alkaloids). If the chemical components of *Trichocereus* might cause poor assimilation of phytosterols (Schreiber, 1958; Harley & Thorsteinson, 1967) or extra energy expenditure for detoxification (Corio *et al.*, 2013; Padró *et al.*, 2014; Soto *et al.*, 2014, De Panis *et al.*, 2016), our results show that an environment rich in proteins could help to mitigate it. Although we found that > 40% of total phenotypic variation was attributable to genetic differences in both species, phenotypic plasticity was also significant. It is interesting to note the great GEI variation in both species, manly in *D. buzzatii*, again providing evidence of a genotypic dependence on the environmental conditions.

Moreover, major differences in wing shape and almost half of the total variation of Size/DT were explained by the genetic factor, even with the low number of genotypes scrutinized. Our results suggest that natural populations harbour significant levels of genetic variation for phenotypic plasticity related to breeding conditions.

The largest differences for wing shape were observed between flies raised in rearing mediums without any addition (TR-W and OP-W), and most of the remainder of the treatments in females of both species and males of *D. buzzatii*. In addition, *D. koepferae* females showed significant differences between cacti. In agreement with the Size/DT ratio results, in *D. buzzatii* the wing shape was similar among flies that emerged from all treatments with protein. In this regard, it has been shown in *D. melanogaster* that a lack of amino acids may result in decreased body size (Colombani *et al.*,

2003), reduced oviposition (Chippindale *et al.*, 1997) and changes in developmental time (Shingleton *et al.*, 2005; Mirth *et al.*, 2009; Koyama *et al.*, 2013). Given that flies reared in *Trichocereus* without any addition showed lower values of viability (mainly in *D. buzzatii*) and Size/DT ratio and greater differences of wing shape than in *Opuntia* with the same conditions and that the addition of protein buffered the effects of alkaloids in both cacti on the two latter traits, our observations reinforce the hypothesis that *T. terscheckii* is nutritionally poorer than *O. sulphurea*.

Different studies have shown several consequences of breeding this pair of cactophilic flies in their secondary hosts, such as an increase in developmental instability, longer developmental times, decreased wing size, lower reproductive success and impaired viability, among others (Carreira *et al.*, 2006, 2008; Soto *et al.*, 2008). Likewise, it has been shown that the strong selection pressure imposed by the alkaloids of *T. terscheckii* on lines of these species resulted in similar effects to those discussed above and even morphological malformations (Corio *et al.*, 2013; Padró *et al.*, 2014; Soto *et al.*, 2014). Given the fact that these studies did not take into account the nutritional factors and the current knowledge about microorganisms in the necrotic tissues of the cacti (Koch *et al.*, 2015), it is possible that evolutionary inferences based only on the intrinsic properties of cacti and, in particular, on their toxic compounds alone fall short. Summarizing, exposure to alkaloids might interfere with development in early stages, leading to a high larval mortality rate. In contrast, a diet poor in protein might lead to modifications in life history and morphological traits, thereby decreasing fitness in adulthood. In fact, and according to our results, the negative effects of alkaloids might be counterbalanced by the presence of proteins in the rearing medium. Additionally, our study also showed that GEI might be the key to understanding differential host utilization. Indeed, previous studies demonstrated that selection pressure could act as a selective sweep of 'weak' genotypes, and therefore improved fitness traits (Padró *et al.*, 2019). In this regard, considering a speciation scenario, differences in the composition of alkaloids and yeasts between host cacti might impose different selective pressure on flies, and GEI could facilitate their differential adaptation (West-Eberhard, 2003; Sultan, 2017).

In conclusion, we consider that the effect of the hosts in this pair of cactophilic species should be studied further, including their particular microbiomes as a potential nutritional source and the ability of the microbiomes to alter the chemical properties of the plants during decomposition. In this sense, our work on nutritional aspects in relationship to chemicals of the cacti opens a wide range of possibilities for the study of the positive effects of yeasts and their interaction with the effects of alkaloids. It is clear that in this complex model of co-evolution, cactus–yeasts–*Drosophila*, all elements are fundamental and deserve to be considered simultaneously.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Figure S1. Gap curve and standard error generated with 100 simulated replicates for each number of clusters. Dashed vertical line denotes the selected number of cluster value, which is the smallest k such that $\text{Gap}(k) \geq \text{Gap}(k + 1) - \text{SE}(k + 1)$, where SE is the standard error.

Table S1. Experimental design: rearing media where flies were raised (columns) with their respective condition and/or additives (rows). In square brackets, we represent units of protein and alkaloids supplemented or naturally present in the medium. For protein, 1 unit equals 3.45 g of killed yeast per 100 g of cactus; for alkaloids, 1 unit equals 0.4 mL of *Trichocereus terscheckii* alkaloid extract (0.932 g/mL) per 100 g of cactus.

Table S2. Number of flies per isofemale line and sex for the morphological analyses. The first column shows isofemale lines, where DKx is a line of *Drosophila koepferae*, and DBx is a line of *Drosophila buzzatii*.

Table S3. Summary of *K*-means clustering analysis of wing size with three clusters. The first row shows isofemale lines, where DKx is a line of *Drosophila koepferae*, and DBx is a line of *Drosophila buzzatii*.

Table S4. Summary of *K*-means clustering analysis of wing size with six clusters. The first row shows isofemale lines, where DKx is a line of *Drosophila koepferae*, and DBx is a line of *Drosophila buzzatii*.